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Assessment of the Bioactivity of Antibodies against β-Amyloid Peptide in vitro and in vivo

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Key Words
Alzheimer's disease · Immunization · Microglia

Abstract
The accumulation of the β-amyloid peptide (Aβ) is a central event in the pathogenesis of Alzheimer's disease (AD). Aβ removal from the brain by immune therapy shows promising potential for the treatment of patients with AD, although the mechanisms of the antibody action are incompletely understood. In this study we compared the biological activities of antibodies raised against various Aβ fragments for Aβ reduction in vitro and in vivo. Antibodies against Aβ enhanced the uptake of $A\beta_{42}$ aggregates up to 6-fold by primary microglial cells in vitro. The kinetics of $A\beta_{42}$ uptake varied considerably among antibodies. Based on the activity to mediate $A\beta_{42}$ uptake by microglial cells, we identified a bioactive antibody that significantly reduced $A\beta_{42}$ levels in the brains of transgenic mice with neuronal expression of an AD-related mutated amyloid precursor protein. This effect depended on the epitopes recognized by the antibody. Our data suggest that the ability to facilitate $A\beta_{42}$ uptake by primary microglia cells in vitro can be used to predict the biological activity of the antibody by passive immunization in vivo. This protocol may prove useful for the rapid validation of the activity of antibodies designed to be used in immune therapy of AD.

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Introduction

Alzheimer's disease (AD), the most common cause of dementia, is an age-related neurodegenerative disorder that is characterized by progressive cognitive deficits, such as memory loss and a decline in mental abilities. An elevated abnormal level of the β-amyloid peptide (Aβ) in the brain is the key step in the pathogenesis of AD [1–3]. Aβ is associated with the formation of neurofibrillary tangles [4, 5] with impaired synaptic functions and the loss of neurons. Therefore, a major emphasis of AD therapy has currently been on the removal of Aβ from the affected brains.

Both active and passive immunization approaches were effective in reducing the brain Aβ levels in human patients and in AD mouse models expressing AD-causing mutations of amyloid precursor protein (APP) resulting in a massive production of Aβ and age-dependent amyloid plaque deposition [6–10]. In addition, anti-Aβ immuniza-

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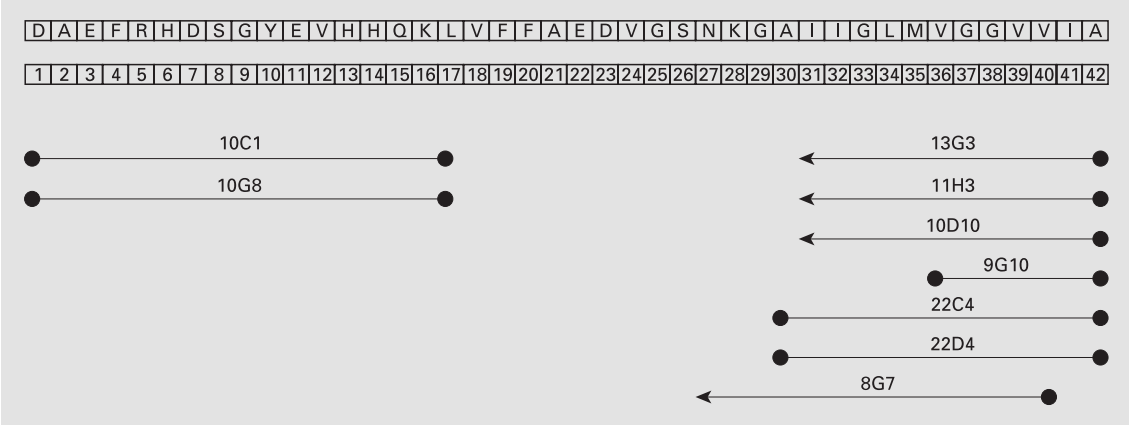

Fig. 1. Schematic presentation of different Aβ fragments that were used to generate the monoclonal antibodies. Letters represent amino acids according to the single letter code. Numbers represent amino acid position within the peptide starting at the N-terminus of the Aβ peptide. Bars correspond to the peptide that was used for immunization to generate the respective antibody. Balls define the ends of peptides used for immunization. Arrowheads indicate that the used peptides reached to the N-terminus of Aβ.

tions exerted beneficial effects on cognitive performances of transgenic mouse models of AD [11, 12] and in human subjects [13], providing the functional evidence for the validity of the Aβ immune therapy. Furthermore, we have recently shown that anti-Aβ immune therapy protected the neurons against apoptotic stimuli in an AD mouse model [10].

Mechanisms implicated in the reduction of brain Aβ by antibodies may include acting as a peripheral sink to reduce cerebral Aβ levels without entering the brain [7]. Alternatively, antibodies may penetrate into the brain, bind the Aβ and interfere with its aggregation [8, 14, 15]. Finally, upon opsonization of Aβ by antibodies, the Aβ/antibody complex is phagocytosed by brain microglial cells in both an Fc receptor-dependent and independent manner [8, 14, 16].

Even though potent in reducing brain Aβ, immune therapy was shown to provoke aberrant autoimmune response such as meningoencephalitis or cerebral hemorrhages in human subjects or mice [17–19], indicating that under certain circumstances, immunization could lead to adverse side effects. Despite intense research and clinical interest in Aβ immunization, the mechanisms by which antibodies result in clearing Aβ from the brain are controversial. It is unclear why some antibodies enter the brain and clear Aβ, whereas some similar antibodies are ineffective in the same system [8] and what the mechanisms of action of the proposed peripheral sink are. Moreover, the immunization protocol and the epitope of Aβ chosen for immunization may affect the outcome of the active immunization [20–22]. Therefore, it is desirable to design a rapid method for predicting the bioactivity of anti-Aβ antibodies by an in vitro test system. In this study the efficacy of microglia cells to take up and degrade Aβ in vitro was used for an initial screening of anti-Aβ antibodies active in vivo. We then verified the bioactivity of anti-Aβ antibodies with a passive immunization protocol in vivo. We assessed the efficacy of these antibodies to reduce brain $A\beta_{42}$ levels and showed that the bioactivity of antibodies against Aβ is dependent on the epitope recognized by these antibodies.

Material and Methods

Hybridoma Cultures and Antibody Production

The monoclonal antibodies were raised against full-length Aβ peptide or its fragments (fig. 1) and the produced antibodies were tested to recognize the respective peptides and the full-length Aβ peptide by Western blotting. Hybridoma clones producing antibodies against various fragments of the Aβ peptide were cultured in OPTI-MEM 1 (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin. The FBS concentration was gradually reduced to a final concentration of 0.1%. Conditioned media were collected and the antibodies were purified via protein A columns (Mo Bi Tech). In brief, the protein A column was washed with 15 ml of 1 × binding buffer (1 *M* glycine, 0.15 *M* NaCl, pH = 8.6) at a flow rate of 1 ml/min. Conditioned media were mixed 1:1 with 2 × binding buffer and pumped through the column at 1 ml/min at room temperature. Columns were then washed with 30 ml of 1 × binding buffer and antibodies were eluted with elution buffer (0.1 *M* citrate,

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pH 5.5 and pH 3), desalted and neutralized by dialysis against PBS overnight. The subclass of purified anti-Aβ antibodies was determined using the monoclonal antibody isotyping kit (Boehringer Mannheim) according to the supplier's protocol.

Primary Microglia Cultures

Primary cultures of mixed glia were prepared from newborn C57BL/6J mice. Meninges, olfactory bulb and hippocampus were removed from brain hemispheres. Neocortical tissue was dissociated by incubation with dissociation solution (0.02% EDTA, 0.24% HEPES, 6.6% trypsin and 0.8% DNase I in DMEM) for 15 min at 37°C followed by an incubation in DMEM (Gibco) containing 10% FBS and 1.6% DNase I for 15 min at 37°C and subsequent triturating by a set of fire-polished glass pipettes of descending diameter. Tissue homogenates were passed through a nylon mesh of 24 μm pore size (Millipore). 5×10^6 cells were plated per T25 flasks and grown in DMEM medium containing 10% FBS to confluency within 10 days. Culture medium was changed after 24 h and every 3 days thereafter. After 14 days microglial cells started to proliferate and floated at the surface of the medium. Loosely attached cells could be mobilized by gentle shaking of the culture. Microglial cells were harvested by aspiration of the supernatant fluid, centrifuged at 100 *g* for 5 min and resuspended in culture medium. For immunocytochemistry, cells were plated on glass coverslips coated with poly-*D*-lysine (30–70 kD, Sigma) at a density of 10,000 cells/cm^2 (low-density cultures). For quantification of Aβ uptake, cells were cultured in 96-well plates at a density of 100,000 cells/well (high-density cultures). Ten minutes after plating, the microglial cells were adherent and nonadherent cells were eliminated by gently rinsing the cultures with medium. Lectin immunostaining identified more than 95% of cells in these cultures as microglial cells.

In vitro Studies

Synthetic human $A\beta_{42}$ (1 mg/ml, Bachem) was fibrillized in PBS as described [23]. For qualitative evaluation of $A\beta_{42}$ uptake in microglial cells by immunohistochemistry, the $A\beta_{42}$ stock was diluted 1:10 with anti-Aβ antibody solutions (300 μg/ml) and incubated overnight at 4°C in a rotary shaker at 5 rpm to generate an $A\beta_{42}$/antibody complex. For quantitative determination of microglial uptake by ELISA, $A\beta_{42}$ stock solution was mixed in a 1:1 ratio with the antibody solutions to generate the $A\beta_{42}$/antibody complex.

$A\beta_{42}$/antibody complexes were diluted 20 or 33 times in culture medium before incubation with low- or high-density cultures, respectively. Three days later the low-density cultures were washed with PBS, fixed with ice-cold methanol for 10 min and subjected to immunocytochemistry. High-density cultures were incubated with $A\beta_{42}$ complexes for 2, 24 or 72 h. After several washes, the cultures were then subjected to formic acid extraction and ELISA measurement of cellular Aβ. Control cultures were incubated with fibrillar $A\beta_{42}$ alone. To control for unspecific binding of complexes to the culture dish, complexes were also incubated in cell-free wells.

Aβ Extraction from Microglia Cultures

High-density cultures were incubated with $A\beta_{42}$/antibody complex or fibrillar $A\beta_{42}$ alone for 2, 24 and 72 h. Cells were washed 3 times with PBS, lysed with 70% formic acid and the resulting extracts were neutralized by addition of 20-fold volume of 1 *M* Trizma base. Internalized microglial Aβ content was quantified by ELISA as described [10, 23]. At least three wells were treated with each antibody in each experiment.

Immunostaining and Western Blotting

After fixation, cultures were washed 3 times with PBS for 5 min and blocked in 10% goat serum for 30 min before incubation with *Griffonia simplicifolia* isolectin B4 (2 μg/ml, Vector) in PBS containing 0.1% Triton X-100 for 48 h at 4°C. After 3 washes with PBS, cells were incubated with anti-lectin (Vector) and anti-Aβ antibodies (4G8, Signet) in PBS containing 1% goat serum and 0.1% Triton X-100 for 2 h at room temperature. For double staining against Aβ and lysosomes, the 4G8 antibody was combined with the anti-lysosomal-associated membrane protein-1 (LAMP-1) antibody (SouthernBiotech). In addition, Iba1 [24, 25] antibody was also used to assess microglia activation.

For analysis of APP processing and microglia activation by Western blotting, mice were anesthetized and perfused transcardially with ice-cold PBS. Frontal parts of the brains reaching from the stereotaxic coordinates interaural 6–5 were homogenized and subjected to Western blotting as described [10, 23]. The remaining parts of the brains were fixed in 4% paraformaldehyde, washed several times in PBS and 35-μm frontal sections were prepared. After electrophoresis, blots were probed with 6E10 (Signet) and C-terminal APP (Sigma) antibodies to study possible alterations of APP processing due to antibody treatments, as well as Iba1 antibody for quantification of the activation status of microglia. Blots were stripped and probed for β-actin (Abcam) as a loading control [10]. Similarly, to test whether the monoclonal anti-Aβ antibodies recognize transgenically expressed Aβ, similar amounts of a brain homogenate of an age-matched SwAPP mouse were loaded onto the gel and probed with the monoclonal anti-Aβ antibodies (50 μg/ml).

Passive Immunization

Transgenic mice with neuronal expression of the Swedish double mutation of APP (SwAPP mice) were bred and housed as described [10, 23]. SwAPP mice were passively immunized by intravenous (i.v.) injections of several monoclonal anti-Aβ antibodies. Each mouse was given four injections into the tail vein of 9 mg antibody/kg body weight at intervals of 5 days beginning at 6–7 weeks of age (n = 3–4 per group) [10]. Mice were sacrificed the day after the last antibody injection. Titers of anti-Aβ antibodies and levels of $A\beta_{42}$ were measured in sera and compared to the corresponding values prior to the injections as previously described [10, 23], and correlated to $A\beta_{42}$ levels in the brains of the same mice. The control group comprised untreated age-matched SwAPP littermates (n = 4).

Statistical Analysis

Data were collected by investigators blinded to the experimental setup and were analyzed by the nonparametric Mann-Whitney U test. In all graphs, means ± SEM are shown.

Results

Microglial Cells Internalized Fibrillar $A\beta_{42}$ and $A\beta_{42}$/Antibody Microaggregates in vitro

To discriminate between antibody-dependent and antibody-independent uptake of $A\beta_{42}$ by microglial cells in vitro, primary microglial cells were incubated for up to 3 days with either fibrillar $A\beta_{42}$ alone or with $A\beta_{42}$/antibody

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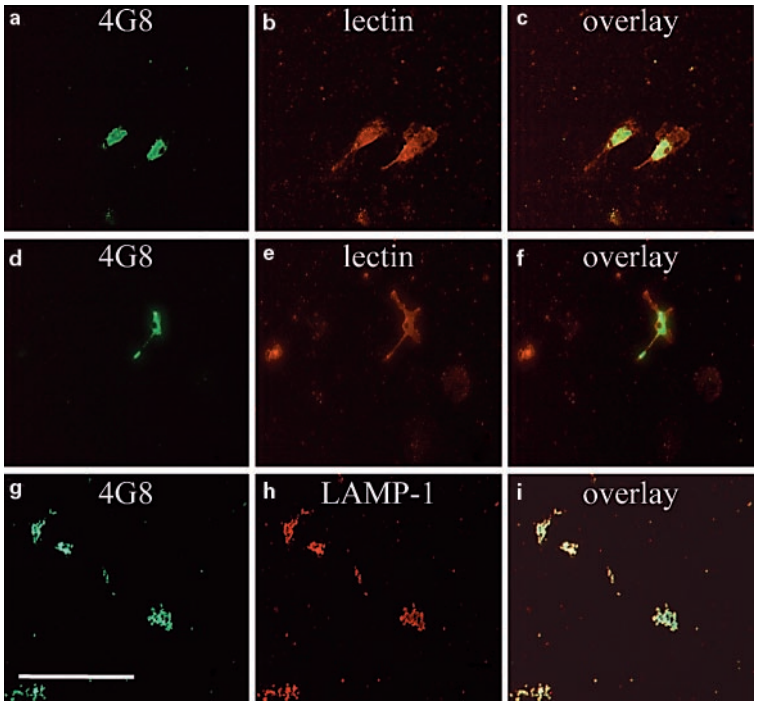

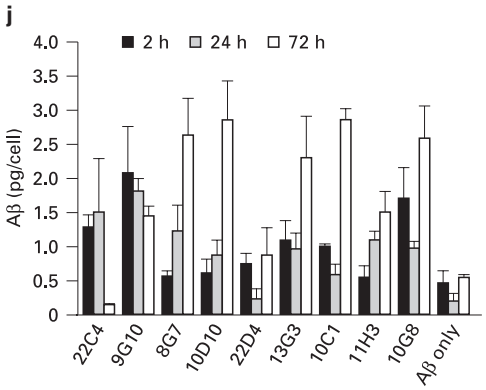

Fig. 2. Accumulation and phagolysosomal localization of $A\beta_{42}$ by microglial cells. $A\beta_{42}$ alone (**a**–**c**) or $A\beta_{42}$/antibody complex (**d**–**i**) were incubated with low-density cultures of primary microglial cells for 24 h and stained with antibodies against Aβ (4G8, **a**, **d**, **g**) and lectin (**b**, **e**) to identify Aβ and the microglial cells, respectively. Microglial cells took up $A\beta_{42}$ both if it was applied as a complex with specific antibodies (**d**–**f**, **g**–**i**) or if applied alone (**a**–**c**). 4G8-immunoreactive material was accumulated in granular structures around the nucleus of microglial cells (**a**, **d** and **c**, **f** overlay). In addition, cultures were doubly immunostained against Aβ and a lysosomal marker (LAMP-1). LAMP-1-reactive structures (**h**) and Aβ-positive structures (**g**) showed a broad overlap (**i**), indicating phagolysosomal localization of Aβ in microglial cells. **j** Quantification and kinetics of $A\beta_{42}$ uptake by microglial cells. Microglial $A\beta_{42}$ levels extracted by formic acid depended on the antibody used and on the incubation time. Measurements were made at time points 2, 24 and 72 h and represented as absolute cellular $A\beta_{42}$ content. Scale 100 μm.

complex of $A\beta_{42}$ with various monoclonal anti-Aβ antibodies generated against the entire Aβ peptide or its N versus C terminal fragments (fig. 1). Immunoreactivity with *G. simplicifolia* isolectin B4 identified the cultured microglia. Cultures were examined by double immunostaining with microglial and Aβ markers. Aβ immunoreactivity was associated with microglial cells, indicating that microglial cells take up fibrillar $A\beta_{42}$ and the $A\beta_{42}$/antibody complexes. Microglial cells accumulated $A\beta_{42}$ both when fibrils were applied alone (fig. 2a–c) and as a complex (fig. 2d–i). To further exclude that $A\beta_{42}$ is only absorbed to the surface of microglial cells but not internalized, cultures were double-stained for Aβ and a lysosomal antigen (LAMP-1). Aβ and LAMP-1 immunofluorescence showed a broad overlap (fig. 2g–i), indicating that double-stained structures correspond to phagolysosomes containing Aβ.

To compare the efficacy of the purified antibodies to mediate uptake of fibrillar $A\beta_{42}$ by microglia, microglia-associated $A\beta_{42}$ was quantified by ELISA in cell extracts (fig. 2j). These measurements revealed a basal antibody-independent uptake of $A\beta_{42}$ by microglial cells. Up to 6-fold higher $A\beta_{42}$ levels were found in microglia cells that were incubated with the $A\beta_{42}$/antibody complex when compared to incubation of the cells with Aβ alone (fig. 2j). Both efficacy and kinetics of $A\beta_{42}$ uptake by microglia cells differed between antibodies used for the formation of $A\beta_{42}$/antibody complexes. Maximum $A\beta_{42}$ uptake was reached after 3 days for most of the complexes, except for 22C4 where the amount of cellular $A\beta_{42}$ was highest at

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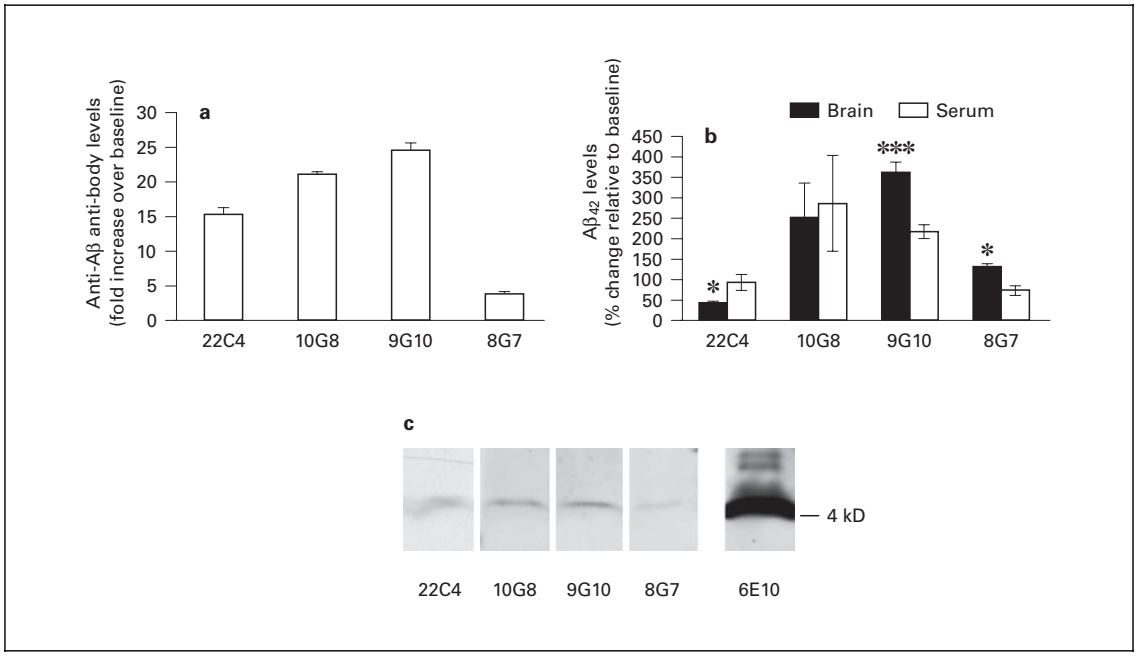

Fig. 3. Passive immunization of SwAPP mice. **a** Elevated levels of anti-Aβ antibody titers were measured at the time of perfusion in sera of all SwAPP mice injected peripherally with identical amounts of purified antibodies. The values are expressed as fold increase, relative to corresponding serum antibody levels of 23 aged-matched untreated SwAPP mice that was set as 100%. **b** $A\beta_{42}$ concentrations in brain homogenates and sera of SwAPP mice injected peripherally with purified anti-Aβ antibodies. The values are expressed in percent relative to a 100% baseline of the corresponding values obtained from averaging readings from a control group of 4 noninjected SwAPP littermates (* $p \leq 0.05$, *** $p \leq 0.001$). Note that no group revealed a significant change of the $A\beta_{42}$ concentrations in serum relative to the baseline. **c** All anti-Aβ antibodies used in the immunization study recognized the human transgenic Aβ expressed in SwAPP brains. The same SwAPP brain homogenate was loaded onto all lanes and the antibody 6E10 served as positive control.

24 h. For 22C4 clone, intracellular $A\beta_{42}$ levels reached basal levels after 3 days, indicative of a rapid uptake of $A\beta_{42}$ and its degradation between 1 and 3 days (fig. 2j). While 8G7 mediated a constant increase of microglial $A\beta_{42}$ over time, 22D4 was completely ineffective in facilitating $A\beta_{42}$ uptake. Thus, this experimental system allows for quantifying the efficacy of antibodies to mediate $A\beta_{42}$ uptake in vitro.

To determine whether the varying effects mediated by different antibodies to take up and degrade $A\beta_{42}$ by microglia cells may be related to different subtypes of IgG, we isotyped the anti-Aβ antibodies used in this study. All antibodies were identified as IgG1-containing κ light chains, thus eliminating the possibility that different antibody subtypes may be responsible for the observed effects.

In vivo Activity of Anti-Aβ Antibodies

The effectiveness of anti-Aβ antibodies to reduce the Aβ levels in vivo was studied by i.v. injection of purified antibodies into the tail vein of SwAPP mice. Hybridoma clones were selected on the basis of their varying kinetics to mediate Aβ uptake by primary microglia in vitro and attention was paid to choose clones generated against defined fragments of Aβ. In addition, one chosen clone recognized only $A\beta_{40}$ but not the $A\beta_{42}$ molecule.

Serum anti-Aβ antibody levels were assessed by analysis of the blood samples taken at the time of perfusion and were compared to baseline antibody levels of 23 untreated age-matched SwAPP littermates (fig. 3a). Antibody titers of the individuals receiving i.v. antibody injections in one group revealed little variation. This finding was expected because a standard amount of purified antibody (adjusted to the body weight of each mouse) was peripherally administrated. Strikingly, even though the same amount of antibody was injected for all groups, the level of antibody detectable in serum was lower for 8G7-injected mice (fig. 3a), probably due to a faster turnover of this antibody in vivo.

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Finally, $A\beta_{42}$ concentrations in sera and brains of SwAPP mice, peripherally injected with purified antibodies, were assessed (fig. 3b). The injection of the antibody 22C4 caused a 55% reduction of brain $A\beta_{42}$ levels ($p \leq 0.05$), while mice injected with 8G7 and 9G10 antibodies showed an unexpected significant elevation of cerebral $A\beta_{42}$ levels. The elevation of $A\beta_{42}$ levels after 10G8 injections did not reach statistical significance (fig. 3b). The levels of $A\beta_{42}$ correlated significantly in the serum and brains of treated mice (Pearson-Spearman correlation coefficient = 0.646, $p \leq 0.05$). For mice injected with the 22C4 antibody, a strong negative correlation was found between the anti-Aβ antibody levels in serum and the $A\beta_{42}$ concentrations in the brain (correlation coefficient: –0.98, $p \leq 0.01$). In addition, i.v. antibody injections did not change serum $A\beta_{42}$ levels in any group (fig. 3b). Moreover, Western blot analysis on SwAPP brain homogenates revealed that all antibodies recognized the Aβ expressed in these brains (fig. 3c).

When compared to untreated brains, no consistent change in APP processing or microglia activation could be detected by Western blotting in brains of mice as a result of passive immunization (not shown).

Discussion

The aim of this study was to set up a system to compare the ability of different monoclonal anti-Aβ antibodies, raised against different fragments of the Aβ peptide, to mediate the uptake and degradation of $A\beta_{42}$ in vitro and in vivo. The results of this study show that antibody-mediated $A\beta_{42}$ uptake by primary microglia cells in vitro can be used as a measure to predict the bioactivity of anti-Aβ antibodies in vivo.

To compare the efficacy at which $A\beta_{42}$ was taken up by the microglial cells when applied as a complex with various antibodies, internalized $A\beta_{42}$ in microglial cells was quantified by ELISA. Different antibodies varied in their activity to mediate uptake of Aβ, both for the overall amount of $A\beta_{42}$ taken up and also for the kinetics of this uptake, indicating that the degradation of $A\beta_{42}$ is affected by the complexing antibody. These variations cannot be explained by differences in antibody subclasses, which are known to differ in their opsonization capacities because all antibodies included in this test belonged to the same subclass.

Active and passive immunization against fibrillar Aβ have proven to be powerful tools in reducing or preventing amyloid pathology in transgenic mice overexpressing human mutant APP [7–9, 11, 12, 15, 21] and in human subjects [6]. It is, however, not yet understood why some anti-Aβ antibodies are potent in removing Aβ from the brain, whereas others are ineffective, even though they recognize Aβ in vitro [8].

Other than by mediating microglial uptake of Aβ, antibodies against the peptide may also clear Aβ from the CNS and plasma by inducing a shift in the equilibrium between the two compartments and facilitate Aβ flux from a central to a peripheral compartment [7, 26]. Moreover, an efficient receptor-mediated bidirectional transport mechanisms for Aβ was shown at the blood-brain barrier that transports the peptide from the CNS to plasma, as well as from plasma to the CNS [26].

Our data are in agreement with studies showing a scavenger receptor-mediated basal Aβ uptake by microglial cells without the action of antibodies and that this uptake is increased by anti-Aβ antibodies [27, 28]. In a similar study, however, an anti-Aβ antibody increased Aβ uptake solely 1.5-fold [27], whereas we observed an up to 6-fold increase of Aβ uptake by microglial cells after antibody treatment. The reasons for the varying capacity of the antibodies to facilitate Aβ uptake may be found in the epitope recognized by the antibody (17–24 on Aβ peptide) and in the antibody subtypes (IgG2) used in that study [27]. Furthermore, passive immunization with an antibody against Aβ of the IgG1 subtype was recently shown to increase the Fcγ receptor expression on microglia in SwAPP mice and led to a reduction of amyloid pathology [29], suggesting an active role of antibodies to promote the clearance of Aβ by microglial cells in vivo.

We chose young SwAPP mice without plaque pathology to assess antibody-mediated changes in soluble Aβ, because concentrations of the soluble Aβ correlate considerably with the degree of cognitive impairment in AD patients [30, 31]. Moreover, we were most interested in $A\beta_{42}$ because it represents the predominant amyloidogenic Aβ species and its levels are massively increased in the progression of pathology in both AD patients and APP transgenic mouse models.

At the time of perfusion, high levels of anti-Aβ antibody titers were measured in all mice injected peripherally with purified antibodies. The antibody level in sera of mice injected with 8G7 was markedly lower, even though exactly the same amounts of antibody had been injected. We postulate that these differences are probably attributable to a variability in the biological activity or antibody turnover in vivo. When compared to untreated SwAPP littermates (baseline), brain $A\beta_{42}$ concentrations were significantly lower in mice treated with 22C4 and unex-

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pectedly increased in mice injected with antibodies 8G7 and 9G10. We could not assess whether the antibodies were able to effectively bind soluble Aβ in the brain. All antibodies used here, however, could recognize Aβ in SwAPP brain homogenates on Western blot level and all have facilitated Fc receptor-mediated microglial uptake of fibrillar $A\beta_{42}$ in vitro, suggesting that they could interact with the Aβ peptide.

The absence of an effect on the brain levels of soluble $A\beta_{42}$ can be explained by the failure of the antibodies to bind Aβ effectively in the periphery and thus exert the effect of a peripheral sink or by the inability to enter the brain and induce enhanced microglial uptake of Aβ. The observed increase of $A\beta_{42}$ concentrations in the brains of mice injected with antibodies 8G7 and 9G10, however, are in stark contrast to most published studies on the peripheral passive immunization with anti-Aβ antibodies, suggesting that anti-Aβ antibodies differ in their ability to reduce $A\beta_{42}$ levels when used in the same experimental setup. Similar increases in brain Aβ levels after anti-Aβ immunization, however, have been shown in other studies [32, 33], indicating again that a shift between Aβ levels of the CNS and periphery is bidirectional and the net effect depends on the antibody used and on the mode of antibody application.

In the present study we show that the bioactivity of anti-Aβ antibodies depended on the epitope recognized. More importantly, the ability of an anti-Aβ antibody to mediate $A\beta_{42}$ uptake by primary microglial cells can predict its bioactivity to reduce the brain levels of $A\beta_{42}$ in vivo. The collected data establish a framework for the evaluation and identification of in vivo active antibodies. In this respect, our data will help to design further experiments aiming at the refinement of amyloid-lowering strategies in vivo.

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